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OPTIMIZATION OF THE FORMULATION AND DESIGN OF OLIGONUCLEOTIDE-BASED PHARMACEUTICALS FOR THE PURPOSE OF GENE THERAPY

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God says in his Holy Qur'an to his prophet,

"We shall show them our signs in the utmost horizons [of the universe] and within themselves, so that it will become clear to them that this [revelation] is indeed the truth. [Still,] is it not enough [for them to know] that your Lord is witness unto everything?"

Holy Qur'an: 41:53

To my parents and my kids

ABSTRACT

Oligonucleotides (ONs) are short sequences of nucleic acids which may be used in a therapeutic context to modulate gene expression. According to their target, ONs can be classified into two main classes: antisense ONs which target mRNA and antigene ONs that target chromosomal DNA. In order to be pharmaceutically efficient, both kinds of ONs have to possess enough stability against degrading enzymes and rapid clearance. They must pass the cell membrane, and in some cases the nuclear membrane, and bind with enough specificity and high affinity to their target site to successfully exert their desired effect. In fact, the use of natural nucleic acids as drugs is hindered by both their inherent instability in biological fluids and their highly charged nature, which hampers their cellular uptake. Therefore, research in the field of ON-based pharmaceuticals focuses on two main strategies: chemical modification of nucleic acids to produce analogues with better stability and binding properties, and development of delivery systems to further stabilize the ONs and enhance their cellular uptake.

Splice-switching antisense ONs (SSOs) made of phosphorothioate 2'-*O*-methyl RNA are promising therapeutics for several disorders caused by aberrant splicing. However, as other ONs, their usefulness is hindered by the lack of efficient delivery. In the first study of this thesis, four amino acid modified versions of the well-known polycation polyethylenimine (PEI) were evaluated for the formulation and delivery of SSOs. The formulations were physically characterized via assessment of their particle size and stability and this characterization was then correlated to their splice-correction efficiency after transfection into mammalian cells. Tyrosine-modified PEI (PEIY) was identified as a successful delivery system for SSOs as shown by splice-correction efficiency of 80% measured in HeLa705; a cell-model containing a mutated β–globin intron sequence found in β–thalassemia splicing disorder.

In the second study, a new cell penetrating peptide (PepFect 14) was developed and investigated for the formulation and delivery of SSOs using cell-models for two splicing disorders; β-thalassemia and Duchenne muscular dystrophy. The feasibility of incorporating this delivery system into solid formulations via solid dispersion technique was also demonstrated. The formed solid formulations were as active as the freshly

prepared nanocomplexes in solution even when stored at elevated temperatures for several weeks.

In the third study, PepFect 14 was evaluated for the formulation and delivery of another kind of ONs: short interfering RNA (siRNA) in different cell lines. RNA interference effect was obtained at low siRNA doses with a unique kinetic profile. Solid formulations were then prepared and assessed for their stability in gastric conditions. PF14/siRNA solid formulations showed marked stability after incubation with simulated gastric fluid, which is extremely acidic and contains proteolytic enzymes.

The fourth study of this thesis addressed design optimization of the newly developed antigene ON, Zorro-LNA (Zorro). Here, double-strand invasion was proven as the mechanism by which Zorro binds to duplex DNA. The original Zorro, targeting both strands of the DNA duplex, was made of two ONs connected via a 7-nucleotide linker. In this report, the possibility to synthesize Zorro as a bi-directional single-stranded ON was investigated, thus reducing the size, facilitating the design and improving Zorro efficiency.

In conclusion, this thesis has dealt with developing formulation strategies for two different types of ON-based pharmaceuticals; SSOs and siRNA. Optimizing the design of Zorro LNA as an antigene ON has been also investigated. These findings may represent a step in the development of ON-based drug products as a new class of therapeutics.

LIST OF PUBLICATIONS

This thesis is based on the following articles:

I. **Zaghloul EM**[*], Viola JR[*], Zuber G, Smith CIE, Lundin KE. **Formulation and Delivery of Splice-Correction Antisense Oligonucleotides by Amino Acid Modified Polyethylenimine**. Molecular Pharmaceutics 2010; 7(3): 652-663.

II. Ezzat K, El-Andaloussi S, **Zaghloul EM**, Lehto T, Lindberg S, Moreno PMD, Viola JR, Magdy T, Abdo R, Guterstam P, Sillard R, Hammond SM, Wood MJA, Arzumanov AA, Gait MJ, Smith CIE, Hällbrink M, Langel Ü. **PepFect 14, a novel cell-penetrating peptide for oligonucleotide delivery in solution and as solid formulation.** Nucleic Acids Research 2011; 39 (12): 5284-5298.

III. Ezzat K, **Zaghloul EM**, El-Andaloussi S, Smith CIE, Langel Ü. **Cell-penetrating peptide mediated uptake of siRNA in solid formulation and its stability in simulated gastric conditions.** *In Manuscript*

IV. **Zaghloul EM**, Madsen AS, Moreno PMD, Oprea II, El-Andaloussi S, Bestas B, Gupta P, Pedersen EB, Lundin KE, Wengel J, Smith CIE. **Optimizing anti-gene oligonucleotide 'Zorro-LNA' for improved strand invasion into duplex DNA.** Nucleic Acids Research 2011; 39 (3): 1142-1154.

Other publications of the author not included in this thesis:

V. Lundin KE, Simonson OE, Moreno PM, **Zaghloul EM**, Oprea II, Svahn MG, Smith CIE. **Nanotechnology approaches for gene transfer.** Genetica 2009; 137:47-56

VI. Lehto T, Simonson OE, Mäger I, Ezzat K, Sork H, Copolovici D,Viola JR, **Zaghloul EM**, Lundin P, Moreno PMD, Mäe M, Oskolkov N, Suhorutšenko J, Smith CIE, EL-Andaloussi S. **A peptide-based vector for efficient gene transfer in vitro and in vivo.** Molecular Therapy 2011; 19 (8): 1457-1467.

VII. El-Andaloussi S, Lehto T, Mäger I, Aizman KR, Oprea II, Simonson OE, Sork H, Ezzat K, Copolovici DM, Kurrikoff K, Viola JR, **Zaghloul EM**, Sillard R, Johansson HJ, Hassane FS, Guterstam P, Suhorutšenko J, Moreno PMD, Oskolkov N, Hälldin J, Tedebark U, Metspalu A, Lebleu B, Lehtiö J, Smith CIE, Langel Ü. **Design of a peptide-based vector, PepFect6, for efficient delivery of siRNA in cell culture and systemically in vivo.** Nucleic Acid Research 2011; 39 (9): 3972- 3987.

CONTENTS

LIST OF ABBREVIATIONS

Ago2	Argonaute 2
Bcl-2	B-cell lymphoma 2
BHK cells	Baby Hamster Kidney cells
BMD	Becker Muscular Dystrophy
CPP	Cell-penetrating peptide
DC-cholesterol	Dimethylaminoethanecarbamoyl cholesterol
DLS	Dynamic Light Scattering
DMD	Duchenne Muscular Dystrophy
DMEM	Dulbecco's Modified Eagle Medium
DOGS	Dioctadecylamidoglycylspermine
DOPE	Dioleoyl phosphatidylethanolamine
DOTAP	Dioleoyloxytrimethylammoniopropane
DOTMA	Dioleylpropyltrimethylammonium cholride
DSI	Double-strand invasion
EDTA	Ehylenediaminetetraacetic acid
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HEK cells	Human Embryonic Kidney cells
HeLa cells	Henrietta Lacks cervical cancer cells
HUH cells	Human Hepatocarcinoma cells
HIV	Human immunodeficiency virus
HPRT1	Hypoxanthine phosphoribosyl transferase
LNA	Locked Nucleic Acid
mRNA	Messenger RNA
miRNA	Micro RNA
NTA	Nanoparticle Tracking Analysis
ON	Oligonucleotide
PAMAM	Polyamidoamine
PEG	Polyethylene glycol
PEI	Polyethylenimine
PLL	Poly-L-Lysine
PMO	Phosphorodiamidate Morpholino Oligonucleotide

PNA	Peptide Nucleic Acid
PS	Phosphorothioate
RES	Reticulo-endothelial system
RISC	RNA Induced Silencing Complex
SCID	Severe combined immunodeficiency
SGF	Simulated gastric fluid
siRNA	Short interfering RNA
SSO	Splice-switching Oligonucleotide
TFO	Triplex forming Oligonucleotide
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

1.1 GENE THERAPY

The concept of gene therapy is based on the use of nucleic acids as drugs for restoring, shutting down or modulating a specific cellular function. In contrast to conventional drugs that often focus on the protein level-treatment of diseases, gene therapy provides a treatment rather upstream at the molecular level of gene expression i.e. on the DNA and RNA levels. It was not until the late 1980s, after the invention of recombinant DNA technology and the development of retroviral vectors, that the first human trials for gene therapy were conducted. In 1990, the first successful gene therapy-based clinical trial was reported when the adenosine deaminase (ADA) gene was transferred into T-cells of patients with severe combined immunodeficiency (SCID) using a retroviral vector (1). Now and after two decades of intensive research in the gene therapy field, scientists have proven that they can treat at least half a dozen rare genetic diseases (Table 1), and that early trials are beginning to find benefits for common diseases as well, including HIV, leukemia, and heart diseases (2,3).

Disorder	Disease Type	Patients benefiting	first publication year
X-SCID	Immunodeficiency	17/20	2000
ADA-SCID	Immunodeficiency	26/37	2002
Adrenoleukodystrophy	Neurologic	2/4*	2009
Leber's congenital amaurosis	Blindness	28/30	2008
Wiskott-Aldrich syndrome	Immunodeficiency	8/10	2010
β-thalassemia	Hemoglobinopathy	1/1	2010
Hemophilia	Coagulation	6/6	2011?

*Includes a patient treated too recently to see benefit

Table 1. Some gene therapy successes over the last decade.

According to the desired therapeutic effect, gene therapy involves two main approaches. The first approach is the restoration of lost gene function via gene delivery using viral vectors or plasmid DNA. The second approach is the silencing or modulation of gene function using shorter sequences of nucleic acids known as "Oligonucleotides" (ONs). It is also worth saying that successful gene therapy basically relies on not only the nucleic acid material to be inserted but also the vector used for its delivery (4). The concept of using ONs for gene modulation or silencing, as well as the optimization of delivery vectors for their successful entry into cells represent the main focus of this thesis.

1.2 OLIGONUCLEOTIDE-BASED PHARMACEUTICALS

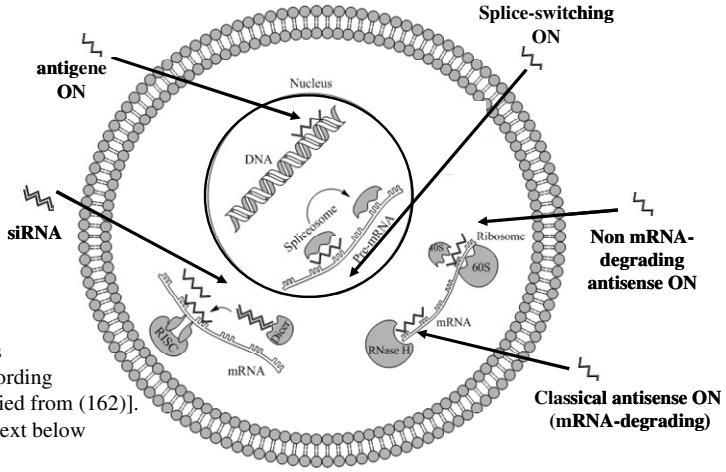


Figure 1. Illustration of most classes of "ON-based Pharmaceuticals" according to their mechanism of action [Modified from (162)]. Detailed description is found in the text below

Oligonucleotide-based pharmaceuticals constitute a new and interesting class of drugs that is rapidly increasing in size and importance. ONs are defined as short sequences (ranging from 8-50 bases) of nucleic acids or nucleic acid analogues that can be used for silencing of targeted genes or modifying their expression pattern. ONs can be classified into two main classes according to their target: antisense ONs which target mRNA and antigene ONs that target chromosomal DNA (5,6) (Figure 1). On their way to exert the desired effects, both kinds of ONs are confronted by many obstacles. They must resist the degradation by serum enzymes and the rapid clearance, they need to reach their target site of action and successfully pass the cell membrane, and in some cases, the nuclear membrane too. In addition, antigene ONs are faced by even more challenges that hamper their action represented in finding their way through chromatin complex structure in order to finally recognize their target sequence within chromosomal DNA and specifically bind to it (7). This may explain the fact that antisense ONs are much described in literature and more explored than ONs working via the antigene approach.

Research in the field of ON-based pharmaceuticals aims at producing ONs with better stability, permeability and binding efficiency (8). Two main strategies have been addressed for achieving these purposes. First strategy is the chemical modification of nucleic acids in order to improve the ONs pharmacokinetic and pharmacodynamic properties. The second approach is the development of delivery systems to enhance the cellular ON uptake. Each of these two approaches will be discussed in its own section under this "Introduction". To start with, antisense and antigene strategies and some of their sub-categories will be described in detail.

1.2.1 ANTISENSE ONs

In 1978, Paul Zamecnik and Mary Stephenson reported the first experiments on antisense mechanisms of gene silencing, using short synthetic antisense ONs to inhibit replication of the Rous sarcoma virus by binding and blocking the action of 35s RNA (9). More recently, the antisense concept was further enriched and broadened by the discovery of RNA interference and of the ability to activate this process using exogenous short interfering double-stranded RNAs (siRNAs) (10,11). Currently, multiple approaches are available for employing ONs to influence the extent and pattern of gene expression via working on the mRNA level of the gene. Antisense ONs bind to their target RNA through Watson-Crick base-pairing and consequently modulate the function of the targeted RNA. In fact, antisense ONs represent a promising drug platform that has the potential to target, in a selective manner, all RNAs in a cell. They can exert their effect either by degrading mRNA (mRNA-degrading antisense ONs) or without degrading mRNA (mRNA non-degrading antisense ONs) (12). The mRNA-degrading ONs include classical antisense ONs, siRNAs, RNA-cleaving ribozymes and microRNAs. ONs belonging to the mRNA non-degrading class work mainly via steric blocking. Examples representing this class include ONs that arrest mRNA translation and ONs that modulate the pre-mRNA splicing patterns. Selected members representing both categories are herein described in details.

1.2.1.1 CLASSICAL ANTISENSE ONs

This class is, in fact, the best understood class of antisense ONs, accounting for the majority of drugs in development. Classical antisense ONs are single-stranded DNA ONs that work through an RNase H-dependent cleavage mechanism. RNase H is a family of enzymes present in all mammalian cells that mediates the cleavage of the RNA in an RNA-DNA hetero-duplex. Human cells contain two types of RNase H: RNase H1 and RNase H2 and both enzymes are thought to play a role in DNA replication and repair (13). RNase H1 is the enzyme responsible for mediating the target RNA cleavage directed by antisense ONs containing five or more consecutive DNA nucleotides (14). The RNase H mechanism has proven to be a robust antisense mechanism and is broadly exploited as both a research tool and a potential human therapeutic. In 1998, Vitravene® (Fomiversen sodium) by ISIS Pharmaceuticals was the first, and so far only, antisense ON approved by FDA. Vitravene® is a classical antisense ON that is administered intravitrealy for the treatment of retinitis induced by cytomegalovirus in HIV patients (15). Other drugs are now in advanced phases in

clinical trials such as Oblimersen sodium; a systemic-acting classical antisense ON developed by Genta Pharmaceuticals and administered by IV infusion for treatment of cancer via targeting the Bcl-2 mRNA (16).

1.2.1.2 SHORT INTERFERING RNAs (siRNAs)

They are ONs that work through another RNA cleavage mechanism known as RNA interference (RNAi). In fact, RNAi is a naturally occurring mechanism in cells used for destroying double-stranded (ds) RNA. This mechanism was first noticed in petunias, when plant biologists attempted to deepen the flowers' purple color by introducing a pigment-producing gene. Instead of intensifying the color, the gene suppressed it and the resulting flowers had white patches or were completely white. A few years later, in 1998, RNAi was recognized by Fire and Mello when they injected a dsRNA into *C.elegans* worms which led to the specific down-regulation of the gene including a sequence similar to that of the dsRNA (17). For this great discovery of the RNAi mechanism, the two scientists were awarded the "Nobel Prize in Physiology and Medicine" in 2006. In 2001, the proof of principle study, by Elbashir and coworkers, showing that exogenous siRNA could achieve sequence-specific gene down-regulation in mammalian cells, marked the birth of siRNA therapeutics (11).

To briefly describe the RNAi pathway: it is initiated by dsRNA which is cleaved by the dicer enzyme into 21-28 nucleotide siRNA duplexes. These duplexes are then transferred to a protein complex termed the pre-RISC loading complex (RNA Induced Silencing Complex) which contains the enzyme Argonaute 2 (Ago 2) (18,19). The pre RISC complex then separates the two strands and an active RISC complex containing only the guide (antisense) strand is produced (20). The active RISC complex recognizes the target mRNA, the guide strand binds via base-base complementarity to it and finally the target mRNA is degraded by the enzyme Ago 2.

Exogenously-delivered siRNA enters this pathway downstream of Dicer (Figure 2).

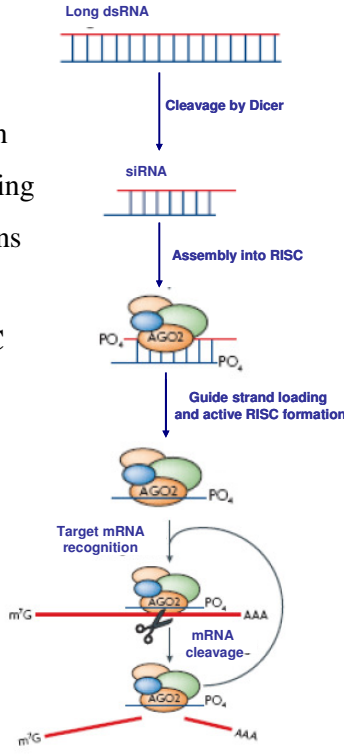


Figure 2. The RNAi pathway and siRNA mechanism of action

What makes the siRNA approach appealing is that the cleavage of target mRNA occurs in a catalytic manner, thus, lower doses are required to achieve gene down-regulation compared to other antisense technologies. That is why intensive research has been carried out to develop siRNA-based pharmaceuticals and a number of them are currently in advanced clinical testing phases (21-25). One example is the ALN-VSP, by Alnylam Pharmaceuticals, which is siRNA in liposomal formulation, designed to target the vascular endothelial growth factor (VEGF) mRNA as a way for treatment of primary and secondary liver cancers (24).

1.2.1.3 SPLICE-SWITCHING ANTISENSE ONs

Splice-switching ONs (SSOs) are antisense ONs that bind to pre mRNA, without degrading it, in order to modulate its splicing pattern. To understand their mechanism of action and their use as pharmaceuticals, some basic facts about the process of pre-mRNA splicing will be introduced.

1.2.1.3.1 SPLICING AND ALTERNATIVE SPLICING.

Pre-mRNA splicing is an essential post-transcriptional process that occurs in the nucleolus of the cell. In this process, pre-mRNA introns are excised and the exons are joined in order to form a typical mRNA to be transported to the cytoplasm and finally translated into protein**.** Frequently, a single pre-mRNA transcript can be spliced to produce multiple mRNA variants which in turn will be translated to different proteins. This process is known as "Alternative Splicing" (26). Factors such as tissue, stimulus and developmental stage were proven to control the process of alternative splicing (27). Interestingly, recent studies indicate that 70% of human pre-mRNA undergoes alternative splicing. This is most probably the major source for protein diversity generating the huge number of proteins present in human cells which greatly exceeds the number of their encoding genes (28).

Splicing and alternative splicing depend on essential sequence elements, known as the "splicing code", in pre-mRNA (29). The splicing code is briefly represented in: 5' and 3' splice sites (at the exon-intron junctions), branch point, polypyrimidine tract and additional splicing regulatory elements that exist within both exons and introns known as exonic/intronic splicing enhancers and exonic/intronic splicing silencers. Components of the basal splicing machinery bind to splice site sequences and promote assembly of the multi-component splicing complex known as, the spliceosome that

catalyzes the removal of introns and joining of exons (30). Alternative splicing can be performed through several different pathways (31) such as those showed in Figure 3.

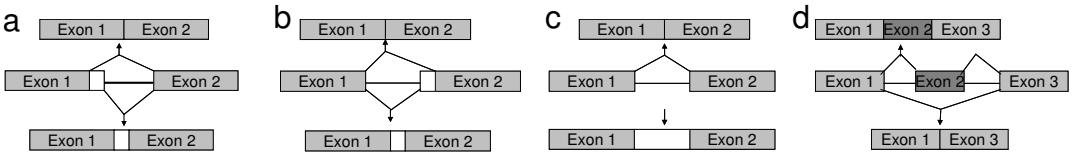


Figure 3. Examples of alternative pre-mRNA splicing pathways. (a) use of alternative 5' splice sites; (b) use of alternative 3' splice sites; (c) intron retention and (d) a choice to include or skip an exon

1.2.1.3.2 ABERRANT SPLICING AND DISEASES

It has been estimated that up to 50% of human genetic diseases arise from mutations that affect splicing (32). Several serious genetic disorders including β-thalassemia , X-linked agammaglobulinemia (33) , cystic fibrosis, atherosclerosis, muscular dystrophies, spinal muscle atrophy and many types of cancers are associated with mutations that alter the splicing pattern (34). One of the first described splicing mutations was found in β-thalassemia patients, where point mutations in intron 1 or 2 of β-globin pre-mRNA creates an apparent 5' splice site which concomitantly activates a cryptic 3' splice site. This in turn leads to inclusion of a part of the intron (a pseudo exon) encoding for stop codon in the mRNA transcript and thus formation of truncated non-functional β-globin protein (35). Several examples of this pathogenic mechanism have been shown for diseases such as cystic fibrosis, ataxia telangiectasia, neurofibromatosis type I and many inherited metabolic diseases (36-39). The well-known muscular disorders "Duchenne muscular dystrophy" (DMD), characterized by progressive degenerative myopathy, and its milder allelic disorder, Becker muscular dystrophy (BMD), are both caused by mutations in the dystrophin gene. In DMD, the mutations result in premature termination of translation and thus lack of dystrophin protein, while in the milder form (BMD), a mutation within a regulatory sequence for splicing leads to in-frame skipping of one or more exons resulting in the formation of a truncated, partially functional protein (40).

1.2.3.1.3 ANTISENSE ONs FOR SPLICE-SWITCHING

A therapeutic platform that has gained increasing attention since the initial discovery in 1993 is the use of antisense ONs to modulate splicing patterns as a way for the treatment of aberrant splice disorders (35). Antisense SSOs bind by complementarity to a selected region in the pre-mRNA and inhibit by steric hindrance the recognition of that region by the splicesomal machinery, thus switching the splicing towards the

desired pattern (Figure 4). Hundreds of studies have investigated the clinical interest of this technology (41-43) therefore; two examples of targets showing great advance in clinical trials will be further described.

β-thalassemia is a genetic blood disorder characterized by a deficiency of β-globin chains, leading to the reduction or absence of adult haemoglobin and thus excessive destruction of red blood cells. It is a fairly common disease and its current therapy consists of frequent blood transfusions combined with iron chelation treatment. The only cure, bone marrow transplantation, is limited by the scarcity of suitable histocompatible donors (44). The use of antisense SSOs that can block the intronic cryptic splice site has greatly succeeded in correcting the aberrant splicing back to normal in erythroid cells restoring β-globin expression (45). Moreover, repair of β-globin pre-mRNA rendered defective by the same splicing mutation was accomplished *in vivo* in a mouse model of β-thalassemia via systemic delivery of antisense SSO conjugated to a peptide (46).

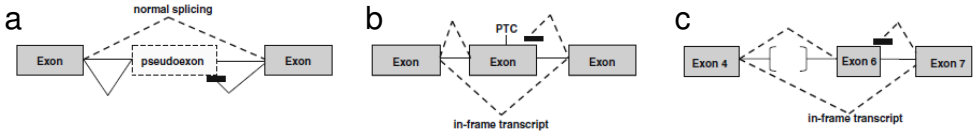


Figure 4. Overview of some of the reported antisense applications for splicing modulation. SSOs (thick black bars) have been used to (a) revert aberrant splicing caused by activated pseudoexons, (b) exclude exons with pretermination code (PTC) mutation, (c) exclude one or more exons to restore the open reading frame.

DMD is a severe X-linked monogenic neuromuscular disorder that affects 1 in 3500 boys worldwide with no radical cure yet (47). SSOs have been applied to promote exon skipping as another mechanism by which splicing pattern can be modulated. In DMD, this approach was validated using SSOs that blocked pre-mRNA splicing elements, thereby excluding the mutation-containing exon and restoring the reading frame in DMD transcripts thus converting the disease to the milder Becker form. Exon skipping SSOs have been successfully evaluated both in cells and *in vivo* using a DMD mouse model; namely "the mdx mouse" where a non-sense mutation in exon 23 leads to premature termination during translation (48). Interestingly, two SSOs targeting mutations in exon 51 for treating the same disease and developed by AVI BioPharma and Prosensa (together with GlaxoSmithKline) are currently in human clinical trials (phases 1 and 2) in DMD patients treated locally (49,50) and more recently systemically (51).

1.2.2 ANTIGENE ONS

The antigene strategy is based on the sequence-specific targeting of the chromosomal ds DNA, in this way acting upstream of the RNA antisense approach. In fact, developing ONs for antigene purpose confronts several challenges (52). Compounds must be able to enter cells, pass into the nucleus, and bind chromosomal DNA with high specificity. Binding must occur in spite of the already existing base-pairing at the target site and complexation of the genomic sequence with histones, transcription factors, and other DNA binding proteins. Once bound, the association of an oligomer with the chromosome must be sufficiently stable and long-lasting to affect gene expression. These challenges make recognition of DNA more complex than recognition of mRNA and overcoming them requires much investigation on the biochemical properties of nucleic acids and their chemically modified analogues forming the antigene ONs. Ten years after James Watson and Francis Crick published their model of the DNA double helix (53), Karst Hoogsteen reported his finding of another mode of binding that also exists, although rarely, forming a triple helix in chromosomal DNA (54). Antigene ONs were developed to bind to ds DNA either via the Hoogsteen or the Watson-Crick mode of binding (Figure 5).

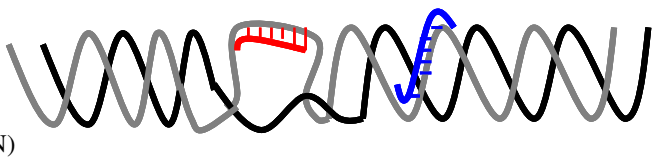


Figure 5. Antigene ONs binding to chromosomal DNA via Hoogsteen (blue ON) or Watson-Crick (red ON) modes of binding. Hoogsteen binding occurs in the major groove of DNA and known to cause little distortion as compared to the Watson-Crick binding, which mostly involves invasion to the duplex DNA. Details are found in the text.

1.2.2.1 BINDING VIA HOOGSTEEN

In the half-century since the first triple helical structures in RNA were reported (55), significant progress has been made in developing triplex forming ON (TFOs) to bind to the major groove of ds DNA and form a triple helix. TFOs may serve as antigene ONs that bind to homopurine stretches in the major groove of duplex DNA via Hoogsteen hydrogen bonds formed between the base of the third strand and the purine base of the duplex DNA (56). The presence of divalent cations Mg^{2+}, Ca^{2+} and Zn^{2+} as well as naturally occurring polyamines such as spermine and spermidine were found to help stabilizing the triple-helix, by reducing the electrostatic repulsion among the three phosphate backbones (57).

Triplex formation occurs in two motifs, distinguished by the orientation of the third strand with respect to the polypurine strand in the duplex DNA (Figure 6). Generally, pyrimidine-rich TFOs bind in a parallel manner, while purine-rich TFOs bind in an anti-parallel orientation (58). In the pyrimidine motif, thymines or protonated cytosines of the TFO bind to A:T or G:C Watson-Crick base pairs, respectively, forming the canonical base triads T:AT and C+:GC. This kind of binding can only occur under acidic conditions, since N3 protonation of the third strand cytosine is required for proper Hoogsteen binding to the N7 of duplex guanine. On the other hand, the purine-rich motif binds to DNA in an antiparallel direction via reverse Hoogsteen bonds to form the base triads G:GC and A:AT. In contrast to the pH requirement of pyrimidine-rich TFOs, effective binding for the purine TFOs requires no protonation, allowing them to bind at neutral pH. However, the guanines in purine-rich oligonucleotides were shown to form G-quartet structures in physiologic concentrations of K^+ (59). This kind of intermolecular complexes could significantly reduce the bioavailability of TFO molecules.

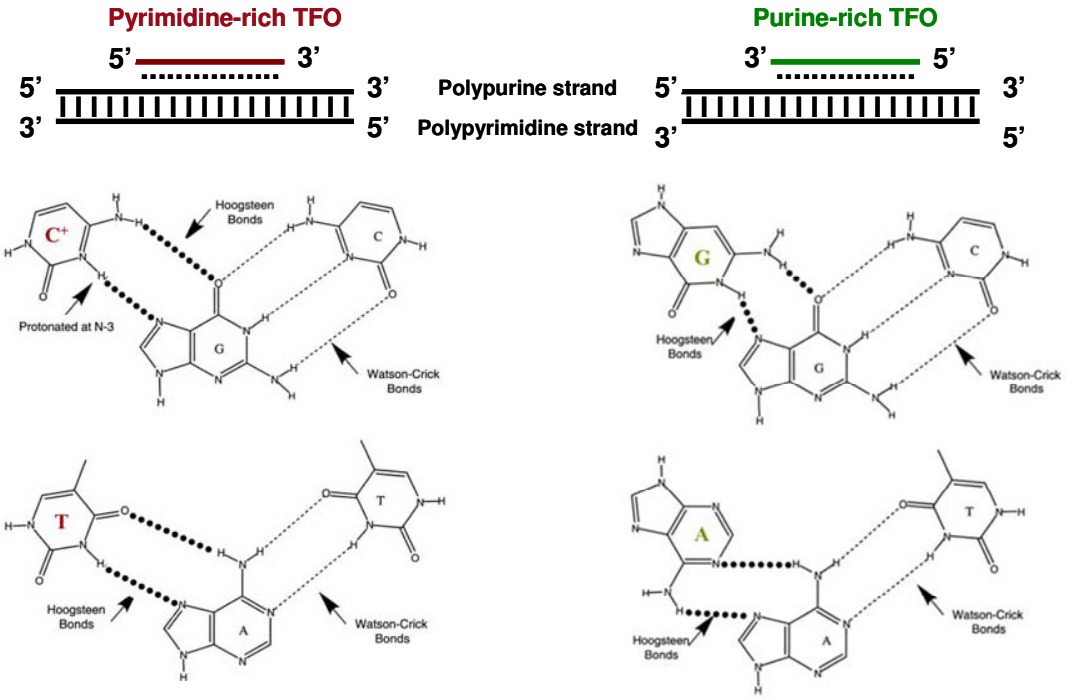


Figure 6. Triplex binding in the purine rich and pyrimidine rich TFOs, [Adapted from (58)].

TFOs have been used to inhibit transcription of some endogenous genes *in vitro* and in cell cultures; however, the efficiencies have not been that great. There are several drawbacks in the TFO technology limiting its use for *in vivo* gene down-regulation. TFOs are sequence-restricted since they are only able to bind to homopurine stretches

in DNA. While triplex formation by TFOs is straightforward under controlled conditions of pH and ion concentrations *in vitro*, the physiological environment in living cells presents substantial obstacles as shown in the low pH required for cytosine protonation and the low concentration of K^+ to avoid G-quartet formation. Moreover, there is evidence that duplex DNA undergoes helical distortions upon TFO binding which could explain the interruption of triplex formation by the nucleotide excision repair machinery in the cell (60)

Many strategies have been investigated in order to optimize the use of TFOs for *in vivo* gene regulation (61,62). Nucleic acids chemical modification, which will be discussed in its own section, has been applied as a main and important one of those strategies (63,64). Polycations grafted with copolymers such as polylysine-graft-dextran have been shown to form a stable association with TFOs which greatly alleviated the electrostatic repulsion between the TFO and duplex DNA and also allowed triplex formation even at physiological pH (65,66). It has been recently shown that using Twisted Intercalating Nucleic Acids (TINA) has a great stabilization effect on the triplex (67). Inserting TINA bases in a TFO has been shown to help avoid the K^+ induced self aggregation problem that usually happens in G-rich TFOs (68).

1.2.2.2 BINDING VIA WATSON-CRICK

ONs that bind to ds DNA via Watson-Crick hybridization mechanism constitute a relatively new and appealing category of antigene drugs. Opposite to TFOs, ONs belong to this class do not require any sequence restriction since, in theory; they can be designed to target any DNA stretch in the genome. Binding by these ONs is performed via invasion into duplex DNA strands: double-strand invasion (DSI). As already referred to in the antisense ONs and TFOs, nucleic acid modifications are, herein as well, required for the sake of getting antigene ONs with good hybridization and efficient DSI properties into chromosomal DNA. Peptide nucleic acid (PNA) and locked nucleic acid (LNA) are the two most common nucleic acid analogues used for making ONs of this class. DSI properties for PNA-based antigene ONs have been clearly investigated (69). DSI associated to transcription inhibition was first reported for a linear PNA-based ON targeted against the CAG triplet of the androgen receptor gene (70). PNA was also found to target specific *c-myc* gene regions (71). Regarding LNA, Corey and coworkers have used linear LNA-based ONs to target progesterone receptor gene transcription start sites leading to gene down-regulation in cell culture

(52,72). However, the actual mechanism of LNA-based antigene ONs was not conclusively demonstrated.

In our lab, we have developed a new LNA-based antigene drug designated “Zorro-LNA”. The original Zorro-LNA (Zorro) consists of two LNA/DNA mixmer ONs attached to each other through a 7-nucleotide (7-nt) complementary linker region. One of the ONs is constructed to bind to the coding strand of DNA and the other to the template strand. It was observed that Zorro can successfully bind to DNA duplexes and inhibit gene transcription on the plasmid level (73). Moreover, when Zorro was microinjected into cells stably transfected with the target site-containing reporter gene, it also mediated blocking of gene expression (74).

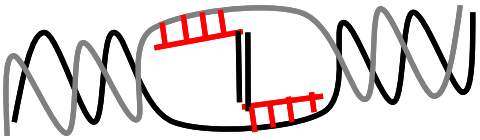


Figure 7. Schematic representation of the original ”Zorro-LNA” concept. Two LNA/DNA mixmer ONs are binding simultaneusly to the sense and antisense strands of DNA. The 2 ONs are connected via complementarity in a 7-nt linker region.

In the fourth paper of the papers constituting this thesis, DSI was proven as the mechanism by which Zorro binds to ds DNA (75). This was the first proof conclusively demonstrating that an LNA-based antigene ON could strand invade into duplex DNA and, as shown in the paper, the Zorro shape was a requirement for achieving DSI. In early 2011, the Zorro concept was used by Hoffman and coworkers where they delivered Zorros into human fibroblasts by microporation to down-regulate the NF1 gene expression (76). Nowadays, in our lab, further optimization of both the design and the formulation of Zorro antigene ONs is currently undertaken for the aim of targeting genes in the genomic DNA context.

1.3 CHEMICALLY MODIFIED NUCLEIC ACIDS (NUCLEIC ACIDS ANALOGUES)

Unmodified DNA and RNA are inherently unstable molecules in biological systems, based on the degradation effect by nucleases that cleave the phosphodiester linkage. This instability hinders the use of natural unstabilized nucleic acids as drugs because they are degraded before they have a chance to reach their target sites of action. In addition, the pharmacokinetics of RNA and DNA make them unacceptable systemic pharmaceuticals because they are weakly bound to plasma proteins thus rapidly filtered by the kidney and excreted into urine. Binding to intracellular DNA or RNA targets, which are known to be highly structured, requires ONs with not only high affinity but also high specificity to avoid the adverse effects resulting from the off-target binding. Collectively, the intrinsic binding affinity and pharmacokinetics of natural ONs are insufficient for their use as systemic drugs (12). Therefore, research dealing with the chemical modification of nucleic acids has got great attention among work done in the field of ON-based pharmaceuticals. These modifications can be classified according to their location within the nucleic acid chain. Nucleic acid analogues can be synthesized via chemical modifications in the phosphate backbone, the sugar, the sugar-phosphate backbone and in the heterocycle (nucleotide base). Examples representing each class will be discussed in this section.

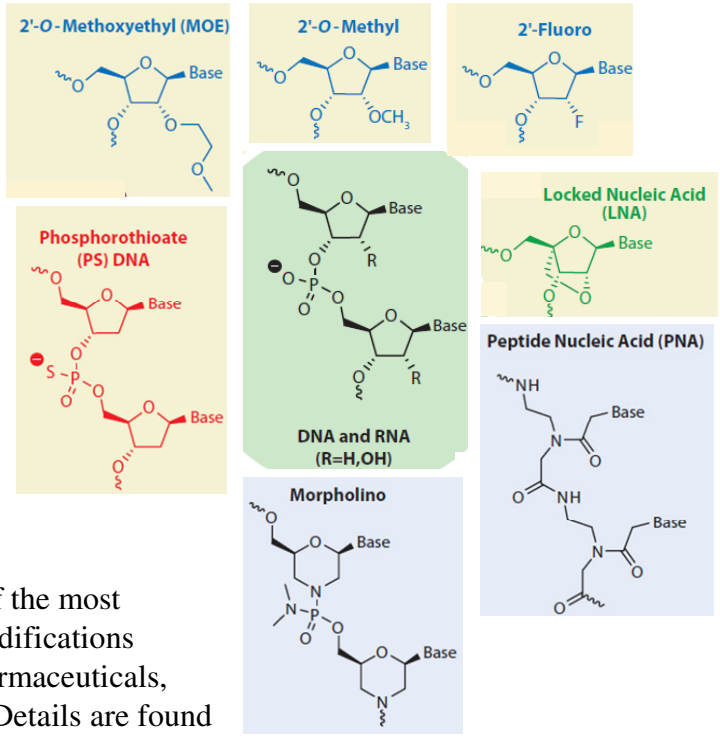


Figure 8. Examples of the most common chemical modifications used in ON-based pharmaceuticals, [Adapted from (12)]. Details are found in the following text.

1.3.1 PHOSPHATE BACKBONE MODIFICATIONS

Since the phosphodiester linkage in natural nucleic acids is significantly susceptible to degradation by nucleases, the ON backbone presents the first target for improvement with chemical modification. Consequently, extensive research has focused on efforts to find backbone modifications that increased the nuclease resistance with maintaining or even improving the affinity and specificity to the target. Phosphorothioate (PS)-containing ONs were one of the earliest backbone modifications where one of the non-bridging phosphate oxygen atoms is replaced with a sulfur atom (77). This substitution provides the ONs with several properties that are crucial for their use as systemic drugs. First, and most important, it greatly increases the resistance to nucleolytic degradation; thus the ON will possess sufficient stability in plasma, tissues and cells and avoid metabolism before reaching to its target. Second, PS modification confers a substantial pharmacokinetic benefit by increasing the binding to plasma proteins; thus increasing the ON circulation residence time and preventing the rapid clearance, which in turn will improve the ON availability to the different tissues (78). Third, PS-modified ON is still able to elicit mRNA cleavage via RNase H activation therefore, it can be used as antisense ON working via the mRNA degrading mechanism. Most antisense ONs that are in clinical trials are PS-modified ONs, also so called "first generation antisense drugs", and the only ON approved by FDA (Vitravene®), as mentioned earlier, is a 21mer PS-modified RNA ON (15). The major disadvantage with PS-modified ONs is their unspecific binding to plasma proteins which might raise toxicity concerns (79,80). Another problem is their reduced affinity to their target RNA; however this is compensated by their high binding specificity. Nevertheless, PS remains the most successful modification to date in ON-based pharmaceuticals. They are used for antisense ONs working by different mechanisms mainly those working by RNase H activation and ONs used for splice modulation (81-83) .

1.3.2 SUGAR MODIFICATIONS

The two most common and extensively studied approaches for chemically modifying the nucleic acid sugar are via substitution on the 2'-position or locking of the sugar ring. Modifications on the 2'-position of the sugar moiety, also known as "second generation antisense ONs", were shown to provide the ON with more affinity and less toxicity compared to the PS-modification described before. Furthermore, the proximity of the 2'-substituent to the 3'-phosphate in an ON generally makes 2'-substitued ON

show higher nuclease resistance (12). Unfortunately, the 2'-modified ONs do not have the ability to activate the RNase H degradation of mRNA. This limitation has been minimized by use of a gapmer strategy, where a block of 2'-modified residues used for increasing the stability and affinity is flanking a central unmodified DNA region of the ON. Otherwise, 2'-modifications have been extensively used in synthesis of siRNAs and SSOs where RNase H-mediated mRNA degradation is not required for accomplishing the effect.

2'-*O*-methyl is the most well-known example representing this category. It has been used for making many splice-switching ONs, currently in clinical trials, such as PRO-051 used for DMD treatment (84,85). Among the 2'-class of modifications, 2'-fluoro has shown the highest binding affinity for target RNA (86). It has been employed by Alnylam Pharmaceuticals in design of siRNAs providing duplexes with increased stability and potency (23). A third example is the 2'-*O*-methoxyethyl (MOE) modification, which is currently the most advanced of the 2'-modified series and has entered clinical trials for multiple indications. It increases Tm by about +2 °C per modification and increases resistance to nucleases. It also appears to reduce the nonspecific protein binding which can reduce toxicities (87). This modification has been mainly pioneered, by ISIS Pharmaceuticals, in the synthesis of many therapeutic antisense ONs indicated for cardiovascular, metabolic, inflammatory and neurodegenerative disorders as well as cancer and currently in clinical trials (87-89). Some examples are Mipomersen (phase III) for treatment of hypercholesterolemia via targeting of apolipoprotein B-100 mRNA (90) and OGX-011 (phase III) targeting clusterin mRNA for prostate cancer treatment (91).

The sugar modification showing the largest known improvement in binding affinity has been made via locking the sugar ring in the well-known nucleic acid analogue: "locked nucleic acid" (LNA) (92). In LNA, a methylene bridge is introduced between the 2'-O and 4'-C of the ribose sugar ring. The resulting conformationally restricted, bicyclic moiety leads to higher stability of duplexes formed with complementary DNA and RNA sequences (93). LNA ONs do not support RNase H, thus a gapmer strategy must be employed for ONs designed to work via this mechanism. LNA-modified antisense ONs, similar to 2'-MOEs, have been exploited for numerous indications such as cancer and hepatitis C (94). Santaris Pharma has developed many LNA antisense ONs such as Miravirsen, also known as "SPC3649" (phase II) for the potential treatment of Hepatitis

C via down-regulating miRNA 122 (95). LNA ONs have shown success in altering splicing both in cell cultures and in mouse models (96). In the antigene field, LNA substituted TFOs have shown an increased triplex thermostability and better binding affinity even at neutral pH, which is important for the homopyrimidine type TFO (97). Linear LNA ONs binding via Watson-Crick were tested in cell cultures (52).

1.3.3 SUGAR-PHOSPHATE BACKBONE MODIFICATIONS

This type of modifications is done via replacement of the sugar-phosphate backbone with an isostere. One of these lead to the phosphorodiamidate morpholino ONs (PMOs), which have a morpholino ring as a replacement of the furanose sugar, with a phosphorodiamidate linkage instead of the phosphodiester backbone. Because of the phosphorodiamidate linkage, PMOs are neutral. These modifications are nuclease stable; however they do not activate RNase H. PMOs have been exploited in steric blocking mechanisms such as splice modulation and translation arrest (98). For splice switching, AVI-4658 (phase II) is a PMO developed by AVI BioPharma for systemic treatment of DMD (99).

Peptide nucleic acids (PNAs) are a completely different class of ON analogues that contain a peptide replacement for the sugar phosphate backbone (100). The uncharged nature of PNA molecules highly decreases the electrostatic repulsion in its hybrids with DNA or RNA, thus increasing the duplex thermal stability. However, PNA ONs are poorly water soluble and do not readily cross cell membranes, which hampered their pharmaceutical development. These limitations have been addressed by conjugation with peptides to improve their cellular uptake (101). PNA-peptide conjugates have been used as SSOs for DMD treatment (102). As stated earlier, PNA has also the ability to strand-invade into a duplex DNA.

1.3.4 HETEROCYCLE (NUCLEOTIDE BASE) MODIFICATIONS

Modification in the heterocycle has focused mainly on increasing the binding affinity for complementary target sites (103). The most common example in this class is the 5'-methylcytosine substitution which is used instead of natural cytosine in homopyrimidine TFOs. 5'-methylcytosine has a higher pKa value which facilitates the TFO binding in physiological conditions. Another replacement for cytosine is the pseudoisocytosine which also remains protonated in physiological conditions, and is

particularly effective in TFOs containing consecutive cytosines. In fact, a TFO with pseudoisocytidine substitutions has been shown to target the human *β-globin* gene in living cells, while the equivalent TFO with 5'-methylcytosine substitution did not form stable triplexes at physiological pH (104).

1.4 FORMULATION OF ONs

Numerous modifications, as discussed earlier, have been proposed to improve the pharmacological properties of ONs, for example to improve their metabolic stability or their affinity, or to increase their selectivity in target recognition. However, free ONs are not taken up efficiently by most cell types unless associated with nucleic acid-delivery vectors. ONs, made of natural and/or chemically-modified nucleic acids, are hydrophilic drugs with low permeability and as such categorized under "class III drugs" in the biopharmaceutical classification system (105). This is due to the highly dense charge distribution on nucleic acids as well as most of the nucleic acid analogues, which ON drugs are made of. In fact, the major obstacle to clinical application of ONs remains their poor cellular uptake. Recently, a number of developed chemically-modified ONs have been proven efficient upon intravenous injection. However, the lack of a targeted carrier system means the usage of large amounts of material, turning this treatment into a highly expensive approach; in particular if the goal is to compete in the market with other treatments. Efficient delivery systems should also be able to compact ONs into well-defined particles that are stable upon storage. Therefore, several viral and non-viral vectors have been developed to improve ON delivery. In this section, a number of non-viral delivery systems that are used for ON formulation will be described.

1.4.1 LIPID-BASED DELIVERY SYSTEMS

Lipid-based delivery systems for nucleic acids are the most extensively studied systems, as shown by the great number of commercially available lipid-based transfection reagents. Two main categories of lipid-based delivery systems have been developed. The first was by chemical conjugation between ONs and lipid molecules such as cholesterol, phospholipids or other alkyl chains with the aim of increasing the lipid solubility of nucleic acids in order to improve their cell membrane permeability (106,107). However, using this strategy, although greatly improving the intracellular delivery, is not optimal in providing the ONs with enough protection against serum nucleases *in vivo*. The non-covalent ON/ lipid complexes form the second and most successful set of lipid-based ON delivery systems. According to the charge of the lipid, two subcategories can be here described. ONs can be encapsulated into the aqueous core of liposomes made mainly of anionic and or zwitter-ionic lipids. Oppositely, cationic lipids can be directly complexed with ONs and these kinds of systems are

called “lipoplexes” and sometimes referred as to “cationic liposomes” (108). The later is the most successful delivery system *in vitro* and offers the most commercially available systems. Cationic lipids are made up of a cationic head group attached by a linker to a lipid hydrophobic group. Lipoplexes are formed via electrostatic interaction between the positively charged head group and the negatively charged nucleic acids (109). Avoiding the tedious step of encapsulating the ON into liposomal formulation, lipoplexes are formed by simple mixing of its components. Examples of typical cationic lipids that can be used for this purpose are DOTMA, DOTAP, DC-cholesterol, and DOGS. Because most liposomal formulations are internalized by endocytosis, during which they would fuse with lysosomes, fusogenic lipids such as DOPE are used to enhance the endosomal escape (110). Lipid-based systems are yet not developed for *in vivo* delivery. Because of their aggregation and non-specific binding to plasma proteins, they are often toxic and eliminated from the blood by the reticulo-endothelial system (RES), with a relative immuno-stimulatory effect (111). PEG-lipid derivatives have been employed to prevent the aggregation, stabilize and prolong the circulation time of liposomal formulation (stealth liposomes) (112).

1.4.2 POLYMER-BASED DELIVERY SYSTEMS

Cationic polymers have been successfully studied for their ability to deliver nucleic acid materials into the cells. They can be bound to nucleic acids (mainly DNA) via electrostatic interaction to form a particulate system known as “polyplex” (113). The chemical structure of polycations comprises repeated units that can be easily manipulated by chemical modification to improve the physical and biological properties of the resultant polyplexes. However, opposed to cationic lipids, they are devoid of a hydrophobic domain, and therefore cannot fuse/destabilize the endosome by direct interaction with the endosomal membrane. The first-generation cationic polymers, exemplified by poly-L-lysine (PLL), were quite inefficient in terms of endosomal escape and transfection efficiency. Co-transfection with endosomolytic agents or introducing histidine moieties into its backbone was required in order to accomplish successful transfection with PLL (109). PLL polyplexes were also rapidly cleared from the circulation, thus addition of PEG has been investigated for prolongation of their circulation half-life. On the other hand, second generation cationic polymers such as polyethyleneimine (PEI) and polyamidoamine (PAMAM) can mediate endosomal disruption by acting as “proton sponges”. Among the polycations

currently used for nucleic acid delivery, PEI has the most prominent position (114,115). PEI can exist as linear or branched polymer and in varying molecular weights. Every three atoms is an amine making PEI a highly protonable polymer, which can induce a buffering effect over a wide range of pH. This buffering property enabled PEI to escape from the endosomes due to the proton sponge effect (Figure 9) (109). Briefly, endosomal membranes possess an ATPase enzyme that actively transports protons from the cytoplasm into the vesicles leading to its acidification. Polymers that contain a large number of secondary and tertiary amines such as PEI can buffer the pH, causing the ATPase to transport more protons to reach the desired pH again. The accumulation of protons in the vesicle results in an influx of counter ions causing osmotic swelling and rupture of the endosomal membrane, and thus releasing of the PEI polyplexes into the cytoplasm.

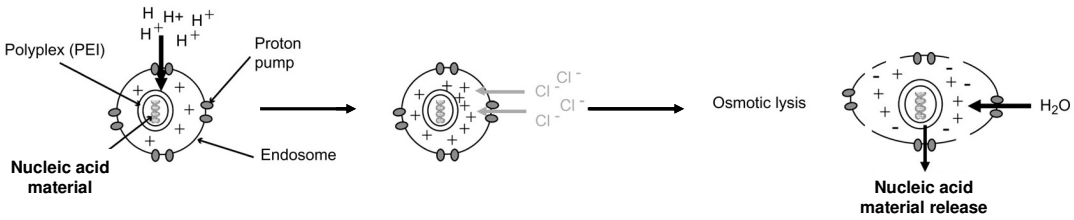


Figure 9. The proton sponge effect as a mechansim of endosomal escape by the polycation "PEI", [Adapted from (109)]

Although being known as the gold standard for plasmid delivery, PEI effectiveness as a vector for ONs is considered to be far less pronounced. This can be attributed to the weak electrostatic cohesion between the polymer and the relatively short ONs, which leads to rapid dissociation of the polyplexes at the anionic cell surface. Moreover, PEI is known to be toxic when used at high doses. This toxicity is associated with the high amount of positive charges that can cause membrane damage in cells and non-specific binding to serum proteins hence rapid clearance by RES before reaching their site of action (116). The non-biodegradability of the polycation is another factor causing its high toxicity. Therefore, modification of PEI and/or its incorporation into other polymers have been recently investigated for getting less toxic derivatives that can be used for efficient formulation and delivery of ONs.

1.4.2.1 MODIFICATIONS IN PEI

The relative simplicity in modifying PEI backbone has been exploited in order to generate delivery systems that are target-specific, less toxic and more efficient for short

ONs. Amelioration of the strong positive charges of PEI, without reducing its "proton sponge" buffering capacity, has been the main goal in this polymer's modification. Hydrophobic modification of PEI has been shown to improve cell membrane interactions for its formulation with siRNA. Recent studies demonstrated that attaching cholesterol to PEI backbone promoted siRNA stability in water-soluble lipopolyplexes and inhibited VEGF expression *in vitro* and induced tumor regression *in vivo* (117). Modification of branched PEI (25 KDa) by aliphatic lipids such as oleic and stearic acid resulted in better condensation and delivery of siRNA into melanoma cells (118,119). Modifying PEI with hydrophobic amino acids has also been reported to produce derivatives with marked efficiency in siRNA delivery into mammalian cells (120,121). In paper 1 of this thesis, PEI modified with the amino acid tyrosine, designated "PEIY", was shown to form stable polyplexes with 2'-*O*-methyl SSO exerting high splice-correction efficiency in HeLa705 cells; a reporter model for β-thalassemia described in the "Methods" section (122). Alternatively, introduction of negative charges into the polymer backbone has been shown to enhance the efficiency and biocompatibility of the polymer (123,124). Examples of the latter was the introduction of succinic acid groups into the backbone or acetylation of the amino groups, which resulted in more stable and less toxic polyplexes with siRNA. Reducing the surface charge of the polyplexes was also achieved via PEGylation (125,126). Successful trials using PEG-PEI copolymers for delivery of 2'-*O*-methyl SSOs in cultured cells and in mdx mice have been reported (127,128).

The non-biodegradability of PEI (25KDa), also known as high molecular weight PEI, made scientists think about synthesizing degradable versions of PEI. Those versions mainly consist of low molecular weight (LMW) PEIs, linked via cross-linkers that can be easily removed inside the cells (129). PEIs in such form displayed much lower cytotoxicity as a result of their rapid in-situ degradation into small molecular weight water-soluble fragments, easily processed and removed by the cells. Interestingly, hydrophobic modification of the LMW PEI further improved the stability of siRNA polyplexes and hence resulted in better down-regulation efficiency (130).

1.4.3 CELL-PENETRATING PEPTIDES (CPPs) AS DELIVERY SYSTEMS.

1.4.3.1 DISCOVERY AND TYPES OF CPPs

The idea of using peptides as delivery systems came from the observation that some proteins, mainly transcription factors, could shuttle within the cell and from one cell to another. A more specific finding was reported in 1988 by two research groups that independently published results in the same CELL issue showing that both the recombinant and the chemically synthesized Tat protein (86 amino acids) were found to be rapidly taken up by the cells in tissue culture (131,132). Few years later, the 60 amino acid homeodomain of the Antennapedia protein in Drosophila was also shown to penetrate cells (133). It was not until 1994 when the cell-penetrating capability could be achieved by a relatively short peptide sequence (16-mer) derived from the third helix of the homeodomain of the Antennapedia and termed "penetratin"(134) . This peptide and others such as: the 11-mer peptide derived from the HIV-1 tat protein, the 27-mer chimeric peptide termed "transportan" and even simple polyarginines (R8) were all shown to translocate across the plasma membrane and carry molecules along in this process (135). During the past few years several other CPPs have been discovered and studied as drug delivery vehicles, some of which are stated in (Table 2).

Protein derived

Penetratin	RQIKIWFQNRRMKWKK-
Tat (48-60)	GRKKRRQRRRPPQ
pVEC	LLIILRRRIRKQAHAHSK-NH_2
bPrPp	MVKSKIGSWILVLFVAMWSDVGLCKKRP KP-NH_2

Chimeric/synthetic

M918	MVTVLFRRLRIRRACGPPRVRV-NH_2
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL-NH_2
TP10	AGYLLGKINLKALAALAKKIL-NH_2
MAP	KLALKLALKALKAALKLA-NH_2
Poly Arg	$(RRR)_n$
Pep-1	KETWWETWWTEWSQPKKKRKV
MPG	GALFLGWLGAAGSTMGAPKKKRKV-

Table 2. Selection of CPPs and their sequences

According to the origin of the peptide, CPPs can be protein-derived such as tat and penetratin or chimeric such as transportan, TP10 and MPG or synthetic CPPs such as the polyarginines (136). CPPs in general can be defined as polybasic and/or amphipathic peptides, usually less than 30 amino acids in length that are internalized within most cell types. In fact, these peptides have recently attracted much interest as

promising non-toxic delivery vectors for different kinds of therapeutics such as small drug molecules, proteins, plasmid DNA and ONs (135,137,138) .

1.4.3.2 FORMULATION STRATEGIES OF ONs WITH CPPs

Two main strategies have been used for attaching a CPP to its cargo: either via a covalent linkage or through the formation of a non-covalent complex. The covalent conjugation approach has been applied to link CPPs to various cargos. Absorption, distribution, tissue targeting and cellular uptake of many drug molecules such as anti-cancers and anti-HIV have been improved via chemical conjugation to CPPs (139-141). In the field of ON-based pharmaceuticals, the covalent coupling of CPPs has been an advantageous route for delivery of uncharged nucleic acid analogues such as PMOs and PNAs. In dystrophin deficient mdx mice, PMOs chemically conjugated to polyarginine-based CPPs could accomplish high levels of splice-switching as shown by restoration of dystrophin protein to more than 50% of normal levels in all peripheral muscles (142). Moreover, an evidence of restored cardiac dystrophin protein was observed for the first time using those CPP-PMO conjugates (98,143). Oppositely, when naked PMOs were intravenously administered to mdx mice, although using high doses and a multiple-injection regimen, the splice-switching efficiency remained relatively low with high variations and no observed effect in the heart (144). PNA-based ONs used for splice-switching have also been conjugated to CPPs resulting in high efficiency in many splice models such as the DMD model and the HeLa705 cells (145). However, coupling of CPPs, which are positively charged, to ONs made of negatively charged nucleic acids has not been as easy to achieve. This limitation plus the laborious procedures involved in the chemical conjugation led to the other approach of linking cargoes to CPPs: the non-covalent complexation.

The non-covalent or co-incubation strategy is mainly based on the electrostatic interaction between the positively charged CPPs and the negatively charged nucleic acids. This strategy has many advantages such as avoiding the time-consuming chemical conjugation and subsequent purification procedures. Moreover, there is no limitation on the size of the ON and interestingly, it was found that lower concentrations of ONs are required to achieve a biological response. Non-covalent complexation was used for efficient delivery of siRNAs both *in vitro* and *in vivo* (146). The amphipathic MPG was the first CPP reported for successful delivery of siRNA targeting GAPDH mRNA after non-covalent complexation (147). Another study

showed that a small peptide derived from rabies virus glycoprotein (RVG) and attached to a polyarginine stretch successfully delivered antiviral siRNA into CNS of Japanese encephalitis virus-infected mice and prolonged their survival rate (148). Another interesting way for formulating siRNA to a CPP was through using a Tat fusion protein with an RNA binding domain (RBD). In that way the RBD domain is used as a molecular glue to attach the Tat peptides to the siRNA (149). This system promoted significant siRNA-mediated gene silencing in different primary cell cultures, however rather high siRNA concentrations were required. The non-covalent strategy has also been applied for the efficient delivery of SSOs made of 2'-*O*-methyl phosphorothioate nucleic acids (150).

1.4.3.3 MODIFIED CPPs AND THE "PEPFECT" PEPTIDES

CPPs are taken up primarily by endocytic pathways, and consequently the cargo is commonly retained in endosomes without reaching their site of action within the cellular machinery (135,151). This obstacle can be overcome *in vitro* using lysosomotropic strategies such as the photochemical internalization treatment or the addition of chloroquine. However, such approaches are not suitable for *in vivo* application. Therefore, several chemical modifications have been introduced to CPPs not only to enhance the endosomal escape and release of cargo but also to improve their membrane interaction and their formulation and transfection efficiency for ON-based drugs (152).

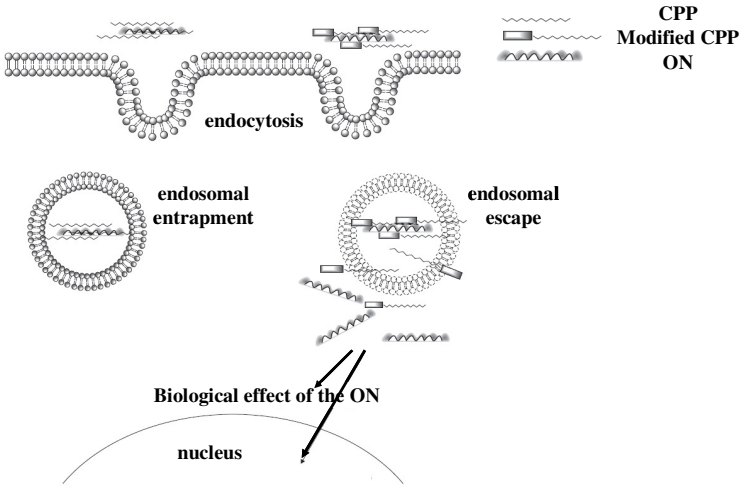
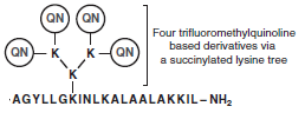
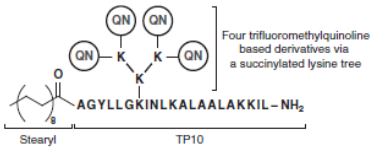


Figure 10.Schematic illustration showing the endocytotic uptake of ON/CPP complexes. Most internalized complexes remain entrapped in endosomal compartments, which greatly reduces the bioavailability of ONs. By introducing different chemical modifications, the release of ONs from endosomes is significantly enhanced, and thus increasing the biological response, [Adapted from (152)].

One example is the development of a histidine-containing endosomolytic α-helical penetratin analogue, EB1, which was able to form complexes with siRNA and promote endosomal escape (153). Another way for avoiding the endosomal entrapments is the use of fusogenic peptides such as HA2 peptide and conjugate them to CPPs (154,155). C-terminal cysteamide modification in MPG and CADY peptides was shown to increase their membrane association and stabilize complex formation by the formation of peptide dimers (147,156). Cholesteryl modification of polyarginines was reported to enhance their efficiency for local delivery of siRNA targeting VEGF in mice bearing subcutaneous tumors (157). Modifying CPPs with lipophilic moieties such as fatty acids has also been employed to increase the bioavailability of non-covalent complexes of CPPs with ONs. The conjugation of decanoic acid moiety to Tat-PNA ONs conjugates significantly increased the splice-correction efficiency by the system (158). Stearylation of the amino acids in a CPP has been shown as another platform for improving the transfection efficiencies of many CPPs for delivery of siRNAs (159,160) and 2'-*O*-methyl PS-ONs (161).

The PepFect (PF) series of CPPs (Table 3), developed by the Ülo Langel group, is based on the chemical modification of CPPs and important members of this series will be discussed here (162).

Table 3. Selected members from the PepFect series of CPPs

Name	Sequence
TP10	**AGYLLGKINLKALAALAKKIL- NH2**
Stearyl TP10 (PF3)	**Stearyl-AGYLLGKINLKALAALAKKIL- NH2**
PF5	
PF6	
PF14	**Stearyl-AGYLLGKLLOOLAAAALOOLL-NH2**

Starting from transportan 10 (TP10), N-terminal stearylation of TP10 produced stearyl TP10 known as PF3. Although being highly efficient for delivering plasmid DNA in

cells and in mice (163), this peptide was shown to have a modest effect for delivery of short ONs such as SSOs in cell models and only in serum-free conditions (161,164). To further improve the endosomal escape efficiency, a trifluoromethylquinoline (QN) - based derivative was conjugated to succinylated Lysine^{7} residue in TP10. This molecule is working as a proton sponge which would greatly improve the endocytic escape of the ON. This derivative, named as PF5, caused a high level of efficiency when used for delivery of siRNA into cell cultures (165). To further improve the stability, both the QN moiety and stearylation, previously used in PF3, were combined, generating the PF6 peptide. PF6 was shown to be highly active in delivery of siRNA into hard-to-transfect and primary cells with quite high tolerability to serum. More interestingly, *in vivo* gene down-regulation was achieved after systemic administration of siRNA formulated with PF6 (165). Another kind of modification in TP10 has led to the development of the new derivative PF14, which is characterized by the high stability in acid conditions, thus enabling the formulation of ON as an orally-administered solid dosage form (166). PF14 is further discussed in paper 2 and 3 of the papers constituting this thesis.

2 AIMS

This thesis describes the use of three different ON-based pharmaceuticals, which are: splice-switching ONs, siRNA and an antigene ON working via Watson-Crick mode of action. The studies included in this thesis collectively aimed at optimizing the formulation and design of these ONs in order to improve their delivery and pharmaceutical significance. More focus was put on the following specific areas:

1- Testing the efficiency of four different modified versions of PEI for the formulation and delivery of splice-switching ONs in mammalian cells.
2- Developing the new CPP "PF14" as a successful delivery system for splice-switching ONs both in solution and as a solid formulation.
3- Evaluating the efficiency of PF14 for formulation and delivery of siRNA in mammalian cells and assessing the gastric stability of its solid formulations.
4- Studying the binding mechanism of the newly-developed antigene ON "Zorro-LNA" and further optimizing its design.

3 METHODOLOGICAL CONSIDERATIONS

Numerous methods were used during this work. In this section, many of them will be discussed with some theoretical aspects.

3.1 SYNTHESIS

3.1.1 SYNTHESIS OF DELIVERY VECTORS.

In this thesis, polymer-based and peptide-based vectors were used for formulation and delivery of ONs. Here is briefly described the synthesis of those vectors.

3.1.1.1 SYNTHESIS OF MODIFIED PEI DERIVATIVES

In paper 1, the well-known polymer PEI was chemically modified with four different amino acids (Tyrosine (Y), Tryptophan (W), Phenylalanine (F) and Leucine (L)) in order to get potentially more efficient delivery vectors for SSOs. Branched 25 KDa PEI containing primary, secondary and tertiary amines in a ratio of 1:1:1 was modified to produce the new derivatives (PEIY, W, F and L). From a synthetic point of view, the chosen amino acids can be coupled to PEI-amines without destroying nucleic acid-binding properties, because over the course of the reaction a cationic amine replaces the reacted one (120). Briefly, the primary amines were allowed to react fully with the esters of butyloxycarbonyl (Boc)-protected amino acids. Boc groups were removed with trifluoroacetic acid, followed by dialysis and finally gave the desired products, with R-amino acid contents of 30% per ethylenimine. PEIY and PEIW were also made with 20% substitution and compared to the ones with 30% modification.

3.1.1.2 SYNTHESIS OF PF14

PF14 is a newly developed stearylated CPP. We have used PF14 as a vector for formulation and delivery of SSO and siRNA in paper 2 and 3 respectively. The methodology known as “solid-phase peptide synthesis”, pioneered by Bruce Merrifield in 1963, has led to great advance in the peptide synthesis field (167). This methodology is based on anchoring the growing peptide chain onto an insoluble solid matrix followed by adding an N-terminally protected amino acid then de-protection and so on. This method is used for the synthesis of peptides, some polymers, DNA and RNA. After PF14 was synthesized, purification was performed by HPLC and the identity of the purified product was verified by analytical HPLC and by mass spectrometer.

3.1.2 SYNTHESIS OF SINGLE-STRANDED ZORRO-LNA

Mixmer LNA/DNA ON strands (single-stranded Zorros) were synthesized by solid phase phosphoramidite chemistry on an automated DNA synthesizer in 1.0 mmol synthesis scale. To achieve strand polarity reversal, i.e. 3'-5'-5'-3' syntheses, we used inverted DNA together with the standard DNA and LNA amidite building blocks, which are commercially available. The inverted LNA amidite building blocks (5'-methyl-C and T derivatives) were synthesized using the recently published procedure by Madsen *et al.* (168).

3.2 OLIOGONUCLEOTIDES FORMULATION AND PHYSICAL CHARACTERIZATION

3.2.1 FORMULATION OF ON/VEHICLE NANOPARTICLES.

In paper 1, we have formulated SSOs using modified PEIs as delivery vehicles. Formulations were prepared via equivolumetric mixing of SSO at the desired concentration with the PEI-derivative at different charge ratios ($NH3^+/PO_4^-$) ranging from 5:1 to 40:1. Both the SSO and PEI-derivatives were first incubated separately in serum-free DMEM medium for 5 min, then they were mixed together and incubated for 30 min at room temperature before transfection to cells or physical characterization.

In paper 2 and 3, SSO or siRNA was mixed with PF14, using different molar ratios, in 50 µl aqueous solution and incubated for 1 hour at room temperature. This was followed by addition of the 50 µl formulation to 450 µl cell medium with or without serum for cell experiments or for physical characterization.

3.2.2 SOLID DISPERSION.

In paper 2 and 3, we made use of the solid dispersion technique to test the possibility of formulating PF14 nanoparticles with SSO or siRNA into solid formulations. Solid dispersion is a pharmaceutical method commonly used to enhance the solubility of poorly soluble drugs by dispersing them in hydrophilic solid matrices (169). Since our nanoparticles were already water-soluble, this technique was used here to produce nanoparticles with uniform distribution over water-soluble excipients by solvent evaporation. Additively, it enabled us to obtain nanoparticles as a solid formulation,

which is considered to be the most stable, desirable and commonly used pharmaceutical dosage form. Briefly, PF14/ON nanoparticles were mixed with solutions of different excipients (mannitol, lactose or PEG 6000) at different concentrations. Then, solvent evaporation was performed by speed drying under elevated temperature (55-60 °C) and reduced pressure.

3.2.4 PARTICLE SIZE MEASUREMENTS

Particle size has always been shown to have a great effect on the efficiency of pharmaceutical formulations. In this thesis, two different methods were used for assessing the particle size.

3.2.4.1 DYNAMIC LIGHT SCATTERING (DLS)

DLS is a well-established technique for measuring the size of molecules and particles typically in the submicron region. Particles in a dispersion system undergo Brownian motion, which is induced by the bombardment by solvent molecules that are moving due to their thermal energy. If the particles are illuminated by a laser, the intensity of the scattered light will fluctuate at a rate that is dependent upon the size of the particles. Analysis of these intensity fluctuations yields the velocity of the Brownian motion and hence the particle size using the Stokes-Einstein equation. The diameter that is measured in DLS is called the "hydrodynamic diameter" and refers to how a particle diffuses within a fluid. This method assumes that the particles are spherical. Measurements are affected not only by the particle core but also by the surface structure and the concentration and type of ions in the solution. In paper 1 and 2, DLS studies were conducted using a Zetasizer Nano ZS apparatus (Malvern). Formulations were diluted in OptiMEM® cell culture medium supplemented with 10% fetal bovine serum (FBS) before measurements. Samples were assessed in disposable low-volume cuvettes. All data was converted to 'relative intensity' plots from where the mean hydrodynamic diameter was derived.

3.2.4.2 NANOPARTICLE TRACKING ANALYSIS (NTA)

NTA is a recently developed technique which allows the tracking of the Brownian motion of nanoparticles in liquid suspension on a particle-by-particle basis. Similar to DLS, NTA measures the Brownian motion of nanoparticles and relate it to particle size through the Stokes-Einstein equation. In contrast to DLS, the Brownian motion is analyzed by visualizing and tracking positional changes of each individual particle in

two dimensions from which the particle diffusion and then the hydrodynamic diameter can be determined. For the analysis of poly-disperse samples or those which contain different particle types of differing refractive index, the NTA approach is far better suited due to its particle-by-particle measurement (170). Because DLS is an ensemble measurement, which is significantly biased to larger, higher scattering particles, the resulting intensity-weighted average can be seriously misleading in the analysis of poly-disperse samples. In paper 3, NTA studies were conducted using the nanosight LM-10HS machine equipped with a sample chamber with a 405 nm laser. Briefly, 0.5 ml of each sample was injected in the sample chamber using disposable syringes. The samples were measured for 40 s with manual shutter and gain adjustments. Both size and concentration of the nanoparticles in each sample was measured by the machine and a 10 sec video clip file showing the particles moving under Brownian motion was taken for each sample.

3.2.5 ZETA POTENTIAL

The particles in a dispersion system have a surface charge resulting from the ionization of surface groups or the adsorption of charged species present in the system. The development of that charge affects the distribution of ions in the surrounding interfacial region, resulting in an increased concentration of strongly bound counter ions close to particle surface forming a first layer known as “Stern layer”. Being strongly attached, it moves together with the particles in the medium. Then, a second layer of ions and counter-ions is formed, which is less strongly bound to the suspended particle. This layer is in direct contact with the bulk of the dispersion system and is known as “diffusion layer”. Thus an electrical double layer is formed around each particle and the potential difference across this double layer is called “zeta potential” (Figure 11) (171). In fact, the magnitude of the zeta potential gives an indication of the potential stability of the colloidal system. Large positive or large negative zeta potential means that there will be enough repulsion between the particles and thus no tendency for aggregation in the system. Oppositely, particles will easily flocculate if they have low negative or low positive or neutral zeta potential. Zeta potential is determined by electrophoresis of the sample and measuring the velocity of the particles using laser Doppler velocimetry. It is highly sensitive to the amount and type of ions in the suspension and its pH. In paper 2, zeta potential of PF14 formulations with SSO was measured in a single use folded capillary cell using the Zetasizer Nano ZS apparatus. It was conducted in a flow cell chamber using the DelsaNano-c (Beckman Coulter) in

paper 3. Measurements were performed in different suspending media: water, OptiMEM® or 0.01mM KCl.

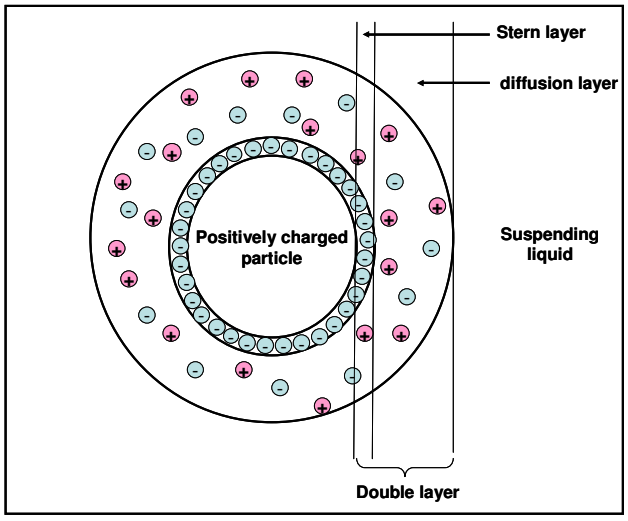


Figure 11. Different layers forming the zeta potential of the particle.

3.2.6 POLYANION COMPETITION ASSAY FOR ASSESSING THE POLYPLEXES STABILITY

One way to measure the relative stability of ON polyplexes is to add a polyanionic compound (such as heparin) which can compete with the positively charged polymer and release the ON from the formulation. The stability of the polyplex is higher when increased concentrations of the competing anion are required. For the polyplex to be ideal, it needs to have an intermediate profile when tested by this method. This means that it should be stable enough to protect the cargo from degradation but not too stable to allow the successful release of the ON to exert its effect. In paper 1, Polyplexes of SSO with PEI or its amino acid modified derivatives were prepared in 20:1 ratio and were incubated for 15 min at 37 °C in the presence of heparin sodium over a range of concentrations. Samples were then analyzed on 1.5% agarose gels and the bands were visualized by staining with SYBR green II.

3.2.7 ACCELERATED STABILITY TESTING FOR SOLID FORMULATIONS

Accelerated stability testing is routinely used to assess the long-term stability of pharmaceutical formulations. In this kind of testing, the formulations are subjected to drastic conditions like high temperature for long time periods. Storage at high temperatures increases the rate of degradation reactions that could take years to occur (172,173). In paper 2, solid formulations of PF14/SSO prepared with lactose at a

concentration of 3.33% were stored either at room temperature or in ovens of adjusted temperatures (40 or 60 °C). Stored formulations were monitored for 8 weeks. Samples were taken at 0, 2, 4 and 8 weeks time-points and then tested for their efficiency in cell culture.

3.2.8 ASSESSMENT OF SOLID FORMULATIONS STABILITY IN SIMULATED GASTRIC CONDITIONS

One of the great obstacles confronting solid formulations is their stability in gastric conditions. In paper 3, we have challenged mannitol solid dispersions of siRNA/ PF14 nanoparticles by incubating them in simulated gastric fluid (SGF) prepared according to the European Pharmacopeia with and without pepsin for 30 minutes. After treatment, the mixtures were either added to cells to verify the efficiency of siRNA in the formulations or analyzed by the NTA system to assess the particle size

3.3 EVALUATION OF FORMULATIONS IN MAMMALIAN CELL CULTURE

Cell and tissue culture models, also known also as (*in vitro* testing), are considered indispensible tools to test the efficiency and toxicity of newly developed drugs. While it can never fully replace animals used for medical testing, it can still help reduce their use. It is relatively low-cost, less cumbersome and it provides researchers with information specifically related to humans. In this thesis, we have used several cell lines to evaluate the efficiency and toxicity of the developed ON-based pharmaceuticals.

3.3.1 CULTURING OF THE CELL LINES

HeLa705, BHK-21 luc, HEK293 luc and HUH7 cell lines were grown in DMEM medium with glutamax and supplemented with 10 % FBS. Cells were maintained at 37 °C, 5% CO2 in humidified incubators. One day prior to transfections, cells were seeded at 50,000 cells per well in a 24-well plate. The H2K mdx myoblasts were grown at 33 °C under a 10% CO_2/ 90% air atmosphere in high-glucose DMEM supplemented with 20 % FBS, 0.5% chicken embryo extract. Mdx mouse myotubes were obtained from confluent H2K mdx cells seeded in gelatin-coated 24-well plates following 2 days of serum deprivation (DMEM with 5% horse serum) and incubation

at 37 °C under 5% CO_2. The myotube development was monitored by light microscopy.

3.3.2 SPLICE-SWITCHING MODELS AND ASSAYS

One of the aims of this thesis was to optimize vectors for successful delivery of the antisense splice-switching ONs. For their assessment, we have used two cell lines representing models of two diseases caused by aberrant splicing.

3.3.2.1 HeLa705 (A REPORTER MODEL FOR β-THALASSEMIA DISEASE)

HeLa705 are cells stably transfected with a luciferase gene in which the coding region is interrupted by a mutated β-globin intron which results in defective splicing and aberrant luciferase protein (174). Masking the mutated splice site by antisense SSO redirects splicing towards the correct mRNA and thus to the corrected luciferase protein. Splice-correction was measured on both the protein and mRNA levels.

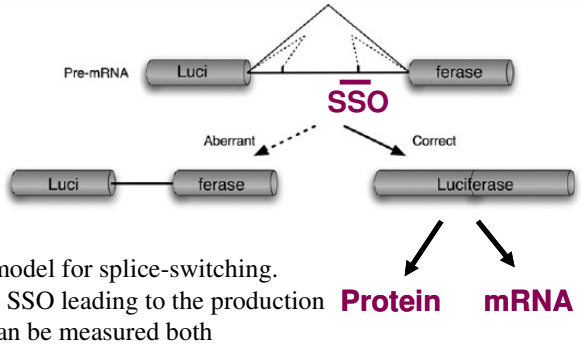


Figure 12. HeLa705 cells as a model for splice-switching. The mutation can be masked by SSO leading to the production of corrected luciferase, which can be measured both on the protein and mRNA levels.

For analysis of the luciferase protein, cells were harvested in Reporter lysis buffer. The protein activity was assessed using luciferase reagent (Promgea) and monitored in Fluostar optima (in paper 1) and in GLOMAX 96 microplate luminometer (in paper 2). For analyzing the corrected luciferase mRNA levels, cells were trypsinized, total RNA was isolated from the cell pellets and RT-PCR was performed. The PCR products were analyzed in a 2% agarose gel in 1x TBE buffer and visualized by SYBR Gold staining. Splice-correction efficiency was calculated as the % of the corrected luciferase band to the sum of the corrected and the aberrant bands.

3.3.2.2 Mdx MOUSE MYOTUBES (A MODEL FOR DMD DISEASE)

Mdx mouse myotubes were obtained from confluent H2K mdx cells, which is a myogenic cell line derived from transgenic mice carrying a point mutation in exon 23

of the dystrophin gene (175). Thus, this cell line serves as the leading cell-model system for development of drugs used for DMD treatment. Masking the point mutation in exon 23 leads to skipping of the whole exon and production of shorter, partially-functional dystrophin mRNA. In paper 2, PF14/SSO nanoparticles were transfected into mdx mice myotubes. RNA was isolated and RT-PCR was performed. The exon-skipping efficiency was calculated as the percentage of the exon-skipping band to the sum of the exon-skipping and the full length bands.

3.3.3 RNAi ASSAYS

For the evaluation of the efficiency of siRNA/PF14 formulations in paper 3, we used two different approaches. First, we used reporter cell lines stably expressing luciferase, transfected them with luciferase siRNA formulated by PF14 and then measured the down-regulation in luciferase expression. The reporter cells used were: BHK-21 and HEK 293 cell lines, both stably transfected with luciferase gene. Luciferase expression was measured, as stated earlier, in the "splice-switching assays" section. Secondly, we investigated the targeting of an endogenous gene that is normally expressed in mammalian cells. We transfected HUH7 cells with hypoxanthine-guanine phosphoribosyl transferase (HPRT1) siRNA formulated with PF14 at different concentrations and measured the effect at different time points. *HPRT1* gene was selected, since its protein has a long cellular half-life (around 48 hrs) so the down-regulation will have a minimal impact on the vitality of the transfected cells. We measured the silencing of HPRT1 on the mRNA level using real-time PCR.

3.3.3.1 REAL-TIME MULTIPLEX REVERSE TRANSCRIPTASE PCR

When it comes to both detection and quantification of gene down-regulation, real-time (also known as quantitative) PCR is the most robust method to do it. In paper 3, we optimized a multiplex real-time PCR allowing both quantification and reliable normalization of the target gene level to the reference gene level in one step. In the same step, the reverse transcription reaction was also included, thus, further increasing the accuracy and minimizing the error. Using this method, isolated RNA was analyzed to amplify HPRT1 (target gene) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (reference gene). The assay was performed using the Quantifast® Multiplex RT-PCR kit. Data were analyzed by the $\Delta\Delta C_t$ method using the StepOne® software version 2.2. Standard curves were made for both genes using known amount of RNA

and serially diluted in order to confirm the efficiency of PCR which was always about 100%.

3.3.4 TOXICOLOGICAL TESTING OF THE FORMULATIONS

Assessment of the potential toxicity of new pharmaceuticals is a quite crucial step usually done in the early phases of drug development (176). Regarding the field of biologic pharmaceuticals, many of the commercial transfection reagents, although showing high efficiency for cell transfection, still have a poor safety and biocompatibility profiles limiting their use for *in vivo* delivery. Cell-based toxicity assays are usually used for screening of drugs and from their results human toxicity can be predicted (177). These assays are based on different mechanisms of action and they evaluate the toxicity of the drug by measuring its response on a specific cellular process or pathway. In this thesis, two different methods were used for toxicity assessment.

3.3.4.1 WST ASSAY

In this assay, cell toxicity is directly correlated to the amount of formazan dye formed as a result from the cleavage of tetrazolium salts by mitochondrial dehydrogenase. Thus, healthy, metabolically active cells have more active enzymes and thus produce more formazan dye which is quantified spectrophotometrically. This method was performed, in paper 1 and 2, according to the manufacturer protocol to assess the cytotoxicity of the different formulations. Briefly, cells were seeded in 96-well plates in DMEM medium with 10% FBS 24 hrs before transfection. Cells were treated with the ON/vehicle formulations or with the vehicle alone at different concentrations in serum-free medium for 4 hours followed by addition of medium with 10% serum for additional 20 hrs. WST reagent was added according to the protocol and then absorbance was measured at 450 nm. The percentage of viable cells was determined by normalizing the values obtained for treated cells with untreated cells.

WST has many advantages compared to other reagents in the family of tetrazolium salts such as the well-known reagent MTT. It is soluble in culture medium, more stable, has a wider linear range and shows accelerated color development. However, this kind of assay, including WST, is a single-point analysis. It also suffers from the lack of quality control when it is used to determine cell viability before and after treatment. These drawbacks are avoided in the newly-developed “XCELLigence” system.

3.3.4.2 XCELLigence ASSAY

This system utilizes an electronic readout called "impedance" to non-invasively quantify adherent cell proliferation and viability in real time. The cells are seeded in standard microtiter plates that contain microelectronic sensor arrays. The interaction of cells with the electronic biosensors generates a cell-electrode impedance response, which indicates cell viability and also correlates with the number of cells seeded in the well. Therefore, this system allows real-time, rather than end point, measurement of cell proliferation, viability and cytotoxicity. PEIY alone and PEIY/ON formulations in paper 1 were evaluated for their cytotoxicity using this system according to the manufacturer's protocol. Briefly, cells were seeded in E-plate and cultured for 20 hrs at 37 °C, 5% CO_2, before the addition of the treatments. Changes in the cell status were monitored and quantified by detecting sensor electrical impedance every 15 min during 45 hrs.

3.4 S1 NUCLEASE DIGESTION

This method was used in paper 4 as a technique to assess the double-strand invasion (DSI) efficiency by Zorro-LNA constructs. S1 nuclease is an enzyme that is known to cleave single-stranded (ss) stretches in ds DNA. Since the binding of Zorro-LNA to ds DNA occurs via DSI, this should create ss stretches that will be accessible to cleavage by the enzyme. While S1 nuclease is specific for cutting ss DNA, it might still cause unspecific nicking and degradation of supercoiled ds DNA under non-optimized conditions. Therefore, in this paper, we adjusted the concentrations and conditions in order to preferentially allow for the specific activity of the enzyme.

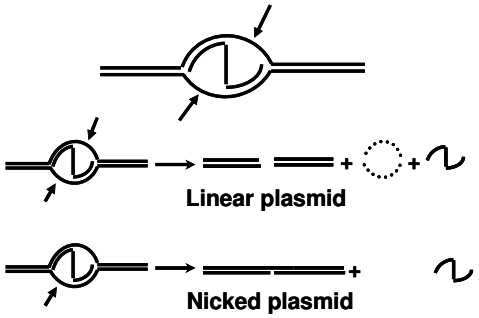


Figure 13. Schematic illustration of how Zorro double-strand invasion will create single-stranded stretches of DNA susceptible to S1 nuclease digestion. As shown by the arrows, the enzyme action will lead to an increase in the nicked plasmid form and/or to a production of the linear form of the plasmid.

Briefly, 0.5 μg of plasmid hybridized with different Zorro constructs or with linear LNA/DNA ONs was digested by 8.3 units of S1 nuclease in 1x S1 nuclease enzyme

buffer. The incubation was done for 4.5 min on ice and the reaction was stopped by adding EDTA to concentration of 77 mM. Samples from the digestion mixtures were analyzed on 0.9% agarose gels and visualized by SYBR® Gold staining. After quantification of the super-coiled, nicked and linear fragments bands, DSI efficiency was calculated based on the ratio between the super-coiled plasmid band and the sum of all bands. DSI C_{50} and DSI t_{50}, which correspond to the concentration (DSI C_{50}) of the Zorro construct and the time (DSI t_{50}) at which 50% DSI happens, were calculated. We used these parameters to compare the DSI efficiency of the different Zorro constructs.

4 RESULTS, DISCUSSION AND PERSPECTIVES

4.1 Formulation and delivery of antisense ONs by modified PEIs

PEI is a well-known polymer that has been the focus of an increasing interest in the study of gene delivery (113,178). PEI is a polymer with a high positive charge density and a high intrinsic endosomolytic activity due to a strong buffer capacity in the pH range between 5 and 7.5 (proton sponge mechanism) (179). Although PEI is known as the gold standard as a plasmid DNA carrier, its effectiveness in ON delivery is considered to be far less pronounced (180). The reason behind that is the short length of ONs, which makes the electrostatic cohesion between the polymer and nucleic acids not strong enough, thus, leading to unstable formulations that easily dissociate at the anionic cell surface. Besides, PEI has toxicity considerations limiting its use for all kinds of nucleic acid delivery.

In this paper, we have modified the commercially available branched 25 KDa PEI with four natural amino acids to produce four derivatives of PEI namely: PEIY, PEIW, PEF and PEIL. We thought that by incorporating hydrophobic amino acids, the polymers would gain a self assembly property, which in turn would enhance the binding efficiency to short ONs. Moreover, these amino acids constitute the core of globular proteins and help maintain protein structure, which would be beneficial in ameliorating the known toxicity of PEI. Finally, we wanted to test these modified PEIs for the formulation and delivery of antisense splice-correction ON, abbreviated in this paper as (ASO), and compare it to what was reported for siRNA delivery. In other words, we wanted to investigate if siRNA (double-stranded, non-modified RNA bases, target site is in the cytoplasm) and ASO (single stranded, 2'-*O*-methyl with PS modified RNA bases, target site is in the nucleus) would behave the same when formulated and delivered into cells using the PEI-derivatives.

DLS studies revealed that both PEIY and PEIW had self-assembly properties, since they could form particles in the absence of the ASO. However, PEIY formed significantly smaller particles with ASO than particles of ASO with PEIW and PEIF. This correlates with many recent reports, which show that providing PEI with mild hydrophobic properties would endow it with the ability to self-assemble, thereby stabilizing the formulation with siRNA (118,120,123).

Interestingly, these modifications in PEI caused a dramatic change in the stability of ASO complexes and resulted in polymers with varied cohesive properties as assessed by the polyanion competition assay. PEIW and PEIF complexes with ASO, upon challenging with heparin, showed very slow release profiles (resistant shell-like particles), whereas with PEIL and the unmodified PEI, the ASO release rate was too fast (quite unstable formulations). This instability was also shown by the DLS studies, where the complexes completely dissociated upon addition of serum-containing medium. Interestingly, PEIY showed an intermediate profile suggesting that the binding was strong enough to protect ASO, but not too tight so it allowed its release to exert the desired effect.

Similar to the study by Creusat G. *et al* (120), PEIY was the derivative that resulted in the highest activity and it significantly enhanced ASO delivery. ASO formulated with PEIY at charge ratio 20:1, or higher, induced 80% splice-correction on the mRNA level in the HeLa705 cells. This correction was significantly higher than that showed by ASO formulated with the commercial lipid-based transfection reagent Lipofectamine® and also exceeded the values measured in other reports using the same cell-model (181-184). Moreover, extremely low doses of ASO (25 nM) still showed significant splice correction; 47% on the mRNA level. When the tyrosine content of PEIY (30% tyrosine/ethylenimine unit) was decreased to 20%, its efficiency was significantly diminished. This indicates that the efficacy of PEIY is also dependent on the degree of tyrosine modification, indicating the importance of keeping a certain hydrophobic/hydrophilic balance for the effectiveness of this polymer.

The other PEI-derivatives were completely inactive for ASO delivery. This can be explained by either the too tight cohesion, which resulted in un-breakable particles (as in the case of PEIW, PEIF) or the weak stability making the particles rapidly dissociate upon contact with the cell surface anions (as in the case of PEIL). Oppositely, when assessed for delivery of siRNA, PEIW and PEIF were shown to be relatively active (120). In that study, PEIW became even more efficient when the tryptophan content was decreased to 20 %. However, the same reduction in the amino acid content did not improve the capacity of PEIW to deliver ASO.

PEIY was not toxic at the doses that showed significant splice-correction efficiency as measured by WST and XCELLigence cytotoxicity assays. If higher concentrations are required for *in vivo* applications for instance, potential cytotoxic effects must be considered. Inclusion of PEG to unmodified PEI and other polymer-based delivery systems has shown improvement in their *in vivo* performance and helped reducing their toxicity (185-187). Similarly, this approach can be used in the future to further optimize PEIY for *in vivo* delivery of short ONs.

4.2 Delivery of SSOs by PF14 and solid formulation development

CPPs constitute very promising tools for *in vitro* and *in vivo* delivery of different types of cargo such as small chemical molecules, nucleic acids and proteins with quite low associated toxicity (135). Endocytosis is the mechanism by which CPPs are thought to utilize for cell internalization. However, one limitation has been their extensive entrapment inside endosomal vesicles. In this paper, we present a new chemically modified CPP, PF14, which builds on previous series of CPPs. Starting from stearyl TP10, we utilized ornithines as the main source of positive charges instead of lysines. The reason behind that exchange was based on earlier reports showing that poly-L-ornithine demonstrated superior transfection efficiency (up to 10-fold) compared to equivalent poly-L-lysine systems. Furthermore, we hypothesized that ornithine, as a non-standard amino acid, would be less prone to serum proteases, and, thus, could retain the activity in serum conditions.

We used PF14 to formulate SSOs targeting splice-site mutations in two cell-line models: HeLa705 cells and mdx mouse myotubes. Robust splice-switching was observed in both cell lines in both serum-containing and serum-free conditions and in a dose-dependant manner. At certain molar ratios, PF14 significantly exceeded the activity of Lipofectamine 2000® without associated toxicity as measured by WST assay. Interestingly, SSO formulated with PF14 elicited a fast onset of splice-switching activity as early as 8 hours.

Uptake of PF14 complexes with SSO was attributed to endocytosis. When we performed transfections in presence of chloroquine, it showed an increase in the splice-switching activity while SSO uptake remained nearly unchanged. The endocytosis mechanism was confirmed by the extensive co-localization of the formulations with labeled dextran; a marker for fluid phase endocytosis.

Then, we wanted to take our delivery system a step further by developing a suitable formulation for administration, more specifically, a solid dosage form, which is considered to be the most widely used form in several pharmaceuticals. We applied the solid dispersion technique by mixing the nanoparticles in suspension with excipient solutions then drying at relatively high temperatures (55-60 °C) under vacuum. The difference in type and concentration of the excipients had a huge impact on the activity of the formulation, with “3.33% lactose” being the one that showed splice-switching

activity nearly identical to the freshly prepared nanoparticles in solution. When the same method using the same excipients was applied to Lipofecatmine 2000® formulations, we found that they completely lost splice-switching activity.

In order to assure the presence of intact particles after the formulation procedure, DLS studies were performed comparing the freshly prepared nanoparticles to the solid formulations. We found that the formulation which mediated the highest splice-switching activity had particle size and particle size distribution most similar to the freshly prepared nanoparticles. Zeta potential measured for the nanoparticles in fresh formulation, or after solid dispersion, had negative values. In fact, this finding raises some interesting questions regarding the mechanism of the internalization and uptake of the particles. In theory, particles with negative surface charge will be repelled by the cell membrane negative potential. However, a previously reported CPP of the PepFect series designated "PF6" showed the same behavior (165). Other reports have also shown that cationic liposome complexes with plasmid DNA are negatively charged under optimal transfection conditions. A very recent study on the uptake mechanism of PF14/SSO nanoparticles has been performed (188). In that study, PF14 uptake has been shown to occur via "class A scavenger receptors", a kind of receptors that mediate the uptake of LDL and many polyanioinic ligands.

Finally, we have run an accelerated stability testing to the best formulation. The study revealed that PF14/SSO solid formulation had an excellent stability profile where no statistically significant loss in splice-switching efficiency was seen at any time-point except for 60 °C after 8 weeks, where the efficiency decreased to 70% of the initial value. Compared to lyophilized lipoplexes (172), PF14 solid formulation without further additives is considered to have an excellent stability profile. Studies in the future may be performed to further optimize the solid dispersion process through usage of other kinds of excipients and other additives that can improve the physical and biological properties of such formulations.

4.3 Development of acid-stable solid formulations of siRNA with PF14

The newly developed CPP, PF14 has shown efficiency in formulation and delivery of SSOs both as solution and as solid formulation. In this paper, we aimed to evaluate the efficiency of the same peptide for formulation of a different kind of ON-therapeutics, namely siRNAs, using the non-covalent complexation strategy. More importantly, we wanted to test the stability of siRNA/PF14 solid formulations in simulated gastric conditions.

First, we used PF14 to formulate siRNA targeting luciferase in HEK 293 and BHK-21 cell-lines stably transfected with luciferase gene. In serum-containing medium, PF14/siRNA nanocomplexes at molar ratio 40 mediated more than 90 % knock-down utilizing 100 nM of siRNA. This shows that PF14 can efficiently deliver siRNA and provide sufficient stability against serum enzymes. In order to challenge the system for a more natural application, we assessed the blocking on the mRNA level of *HPRT1*, as an endogenous gene, in HUH7 cells. In dose-response experiments, the concentration at which 50% of HPRT1 mRNA was depleted (EC_{50}) was 12 nM in serum-containing conditions. This low EC_{50} value indicates the high efficiency of siRNA/PF14 nanoparticles compared to many other delivery systems. Concerning kinetics, PF14 formulations at 50 nM dose of siRNA showed extremely rapid onset of action since the decrease in HPRT1 mRNA reached 70% after only 2 hours. This effect was significantly higher than that obtained by the well-known transfection reagent Lipofectamine RNAimax®. Moreover, down-regulation of HPRT1 mRNA lasted for 4 days, which was the last time-point checked in the experiments.

Similar to the PF14/SSO paper, the solid dispersion technique was herein used for drying PF14/siRNA nanoparticles into solid formulation. Upon screening several excipients using different concentrations, mannitol at the final concentration of 5% was the most optimal. To assure the stability of the nanoparticles after the relatively harsh solid dispersion technique, particle size analysis was performed using the NTA technique. Both measuring the particle concentration and visualizing particles under the Brownian motion are two advantages of the recently developed technique that further confirmed the stability of the formulations. There was no significant difference between the particle size of the freshly prepared formulation and the one as solid dispersion, both showed particle size mean of about 120 nm when measured in OptiMEM® medium with 10% FBS. This obviously assures the stability of the particles after the

speed-drying procedure. To determine the surface properties of the particles, zeta potential was measured both in water and in OptiMEM®. When resuspended in water, the nanoparticles showed a positive zeta potential (22.74 mV). However, when resuspended in OptiMEM®, it was reversed to negative values (-12.98 mV). It is well known that the zeta potential is highly sensitive to the amount and type of ions in the suspension. The net negative charge of the nancomplexes implies that they may recruit the same pathway as that recently reported for those of PF14 with SSO; via class A scavenger receptors (188). Similarly, a part of the siRNA cargo could be displayed on the particle surface at high local concentration enabling such interaction.

Since stability against degradation by the gastric fluid is a crucial factor that affects the bioavailability of drugs after oral administration, PF14/siRNA solid formulations were assessed for such stability. Interestingly, the formulations retained most of their RNAi activity after 30 min incubations in SGF with or without pepsin. When the incubations were performed for longer time periods, the activity was kept the same in the case of SGF incubations with slight decrease in efficiency in the presence of pepsin. This indicates that PF14 effectively protects the encapsulated siRNA from the gastric acidity. The decrease in activity is not surprising for such plain formulation and can be further addressed in the future by other formulation strategies such as coating.

4.4 Zorro-LNA antigene ON: mechanism of binding and design optimization

Zorro-LNA (Zorro) is an antigene ON that was first developed by our group (73,74). The first generation of Zorros (designated 2-ON Zorros) is formed of two LNA/DNA mixmer ONs that are annealed together via complementarity in a 7-nucleotide (7-nt) linker to make a Z-shaped ON. At the first stage of its development, Zorro was hypothesized to strand-invade into duplex DNA, thus binding to the two strands of DNA via Watson-Crick mode of binding but this was never formally proven. In this paper, our first aim was to experimentally prove that Zorro binds by double-strand invasion (DSI) into duplex DNA.

In fact, avoiding intra-molecular binding between bases in the linker region and bases in the arms remained an obstacle, making the design of the first-generation Zorro-LNAs a cumbersome process. Moreover, similar to siRNAs a pre-annealing step was required for the hybridization of the two ONs in order to generate the complete Zorro construct. For an ON-based pharmaceutical, the design should ideally be simple and predictable. Therefore, to make the processes more straightforward, we thought about making the Zorro in the form of a single-ON entity, single-stranded Zorro (ssZorro). In addition, from a size point of view, ssZorro would be smaller than the original 2-ON Zorro. It is well established that small-sized LNA-based pharmaceuticals are more efficient (189). A functional ssZorro would therefore presumably also facilitate its formulation and delivery.

Starting with the first aim of the paper (proving DSI as a mechanism for Zorro-binding to duplex DNA), we made use of S1 nuclease; a restriction enzyme that specifically cuts ss DNA stretches. The digestion conditions were optimized to obtain robust digestion of a plasmid hybridized to the well-known double-strand invader, bisPNA (as a positive control), without any significant digestion of a plasmid hybridized to a TFO (as a negative control). Target plasmid pN25-2BS hybridized with different concentrations of Zorro-LNA showed pronounced digestion by S1 nuclease, while both the mock-treated plasmid and the one devoid of Zorro target sites (pN25-0BS) did not. A second plasmid with a different target site (pN25-tyr), hybridized to different concentrations of the corresponding Zorro showed the same degree of S1 nuclease sensitivity as the hybridized pN25-2BS plasmid. These results clearly suggest that

Zorro-LNAs indeed are able to strand invade into ds DNA without sequence restrictions.

Then, we proceeded to the second aim of the paper, which was the synthesis of ssZorro construct and comparing its DSI efficiency to the original 2-ON Zorro. To investigate if keeping the linker in a double-stranded (ds) form, i.e. in a more rigid form, is required for maintaining the DSI property, we also made a 7-mer LNA-DNA ON (stiffener) to bind to the ssZorro in the linker region. Interestingly, we found that ssZorro is significantly more efficient in DSI into the corresponding target site in the plasmid DNA than 2-ON Zorro. The rigidity does not seem to be of value when it comes to the DSI since the ssZorro without a stiffener gave better results than the one with.

We went one step further by testing the use of other types of linkers than the 7-nt based one i.e. using chemical-based linkers. Application of non-nucleotide linkers has been previously investigated in many ON-based approaches and bioconjugation of ONs with polymers is widely used for improving cell penetration and stability (190,191). Therefore, we synthesized eight ssZorro constructs with eight different chemical linkers that vary in length and hydrophilicity. PEG-based linkers used in the study and the alkyl-chain linker with intermediate length showed prominent DSI. However, the longest alkyl-chain linker (C12 x 3), although having the highest degree of flexibility, showed very poor DSI ability. This could be explained by the hydrophobicity, which could increase aggregation, thereby making this construct less available for strand invasion. Moreover, the flexibility may lead to that the other ON is not ideally positioned for DSI. Since we believe that the smaller the size of the ssZorro, the more feasible will be its formulation and delivery, we also investigated the possibility of using a linker-less construct. Although the later displayed reduced DSI C_{50} and t_{50} values, it eventually reached a high plateau level of DSI, similar to that of the ssZorro with the 7-nt linker. This shows that strand invasion of ds DNA can be achieved, using the smallest (32 nt in length) of all tested Zorros.

To summarize, in this report we have proven DSI as the way by which Zorro binds to duplex DNA. ssZorro was shown to be better in DSI than the original 2-ON Zorro. The composition of the linker could be important for future therapeutic considerations. For future perspectives, we are in the phase of making efficient formulations of Zorro-LNA that can successfully deliver it to the nucleus to exert its action. We are choosing

therapeutic target sites so Zorro technology can be applied for treatment of disease models starting with mammalian cells and then proceeding to *in vivo* animal studies.

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